Cloned Hepatitis Delta Virus cDNA Is Infectious in the Chimpanzee

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A head-to-tail trimer of a full-length cDNA clone of the hepatitis delta virus (HDV) genome was examined for infectivity by direct inoculation into the liver of a chimpanzee that was already infected with hepatitis B virus. Five weeks after inoculation, a marked elevation of serum alanine aminotransferase activity was observed, followed by the appearance of high levels of HDV RNA and antigen in both liver and serum and a high level of viral particles in the serum. A transient suppression of hepatitis B virus replication was evident during the acute phase of HDV infection. Seroconversion for antibodies to delta antigen occurred 3 weeks after the onset of the disease. These results demonstrate that a typical HDV infection can be initiated by inoculation of a susceptible animal with recombinant HDV cDNA.

Hepatitis delta virus (HDV) is a highly pathogenic, hepatotropic agent in humans (32) which requires helper functions of hepatitis B virus (HBV) (28). Experimental transmission of HDV has been accomplished in animals infected by different members of the hepadnavirus family including the woodchuck hepatitis virus in its natural host (24) and HBV in chimpanzees (29). The helper hepadnavirus is either acquired simultaneously with HDV (coinfection) or precedes HDV infection (superinfection) (32). Although the interaction between hepadnaviruses and HDV is not fully understood, one of the helper functions of hepadnaviruses is providing the outer lipoprotein coat bearing the HBV surface antigen (HBsAg) to HDV particles (2, 3, 30). In infected animals, there can be a major suppression of HBV replication at the peak of HDV replication (8, 13, 14, 16, 17, 29, 31), but the mechanism is as yet undetermined.

The cloning and sequencing of the circular RNA genome of HDV has revealed structural and functional similarities to that of plant viroids (11, 15, 19, 21, 43), but unlike the viroids, the HDV genome codes for at least one polypeptide, the delta antigen (HDAg). This protein apparently interacts with HDV RNA and HBsAg in the assembly of 36-nm viral particles (2, 3, 44). The HDV genome, 1.7 kilobases (kb) long, is about four times larger than a typical viroid RNA and has been claimed to share with the latter the presence of a highly conserved region involved in the replication mechanism (4) and the ability to form an unbranched rodlike structure by self-annealing (20, 43). The HDV genome is of antisense polarity and must therefore be transcribed to produce mRNA able to direct the synthesis of HDAg (43, 44). The detection within infected cells of genomic and antigenomic HDV RNAs that are predominantly monomeric with minor amounts of multimeric forms suggests that the genome replication mechanism of HDV is of a rolling-circle type, analogous to that proposed for plant viroids (5, 9). In addition, the ability of the genomic and antigenomic RNA of HDV to undergo self-cleavage and ligation *in vitro* further indicates its functional analogy to plant viroids (20, 36, 37, 47).

Although the replication mechanism of viroids proceeds through an RNA-RNA pathway without DNA intermediates, cloned DNA copies of viroid RNA are infectious when

multimeric forms are introduced into plants by direct inoculation (10, 12). Transcription is initiated from the input DNA template to produce full-length viroid RNA progeny, which in turn initiates the normal replication pathway. This finding has provided the basis for mutational analysis of viroids. In this study, an HBV-infected chimpanzee was inoculated by direct injection into the liver with two recombinant plasmids, one containing a trimeric form of a full-length cDNA copy of the HDV genome and the other containing the HDAg open reading frame only. The synthesis of HDV RNA progeny was initiated, and a characteristic acute, apparently self-limited HDV infection was observed.

MATERIALS AND METHODS

Experimental animal. Alcinda (X136), a 31-year-old female chimpanzee that is an HBV chronic carrier, tested positive for serum HBV DNA, HBsAg, and anti-hepatitis B core antigen antibodies. This animal had no history of previous HDV infection and tested negative for HDV RNA and anti-HDAg prior to experimentation.

Molecular clone. The HDV DNA plasmid pSVL-D3 is a derivative of the eucaryotic expression vector pSVL (Pharmacia, Inc., Piscataway, N.J.), and contains three head-to-tail HDV genomes (18). This vector contains both the simian virus 40 late promoter and a polyadenylation signal and is able to direct the synthesis of a genomic trimeric form of HDV RNA. Another derivative of pSVL (pSVL-Ag), contains the open reading frame coding for HDAg inserted so as to be capable of directing the synthesis of HDAg-specific mRNA (18).

DNA injection. A mixture of 15 µg of both the trimer and antigen clones was dissolved in 3 ml of Williams medium E (Hazelton) containing 150 µg of DEAE-dextran and was injected under ultrasonography at three different locations into the liver of the sedated animal.

Detection of HDV RNA and HBV DNA. Liver biopsies were performed with a Menghini needle on a weekly basis. Total cellular DNA was isolated from liver tissue by digestion with 500 µg of proteinase K per ml in 10 mM Tris hydrochloride (pH 7.4)-10 mM NaCl-10 mM disodium EDTA-0.5% sodium dodecyl sulfate for 2 h at 37°C, followed by extraction with phenol (40). HBV DNA was isolated from serum by the same procedure. DNA samples (5 µg) were subjected to electrophoresis in a 1.5% agarose gel,

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transferred to nitrocellulose by the procedure of Southern (38), and hybridized to 32 P-labeled HBV DNA.

Total cellular RNA was prepared from the same biopsy specimens by disruption in 6 M guanidinium isothiocyanate–5 mM sodium citrate (pH 7.0)–0.1 mM β -mercaptoethanol–0.5% sarcosyl and by centrifugation through a CsCl cushion as described previously (40). Purification of HDV RNA from 100- μ l serum samples was performed by incubation in 50 mM Tris hydrochloride (pH 7.8)–200 mM NaCl–20 mM disodium EDTA–2% sodium dodecyl sulfate–1 mg of proteinase K per ml–2 mM vanadyl ribonucleoside complex for 2 h, and phenol extraction was performed as described previously (27). RNA samples were subjected to electrophoresis through a 1.5% agarose gel in 2.2 M formaldehyde and then transferred to nitrocellulose for hybridization to a 32 P-labeled HDV DNA-specific probe.

Detection and characterization of HDAg polypeptides by immunoblotting. Liver tissue (approximately 1 mg) from each biopsy specimen was disrupted in 200 μ l of sample buffer containing 2% sodium dodecyl sulfate and 2% β -mercaptoethanol, heated at 100°C for 5 min, and then stored at –70°C prior to analysis by the immunoblot procedure as previously described (19). Particles from 1 ml of serum were sedimented through a 20% sucrose cushion at 190,000 $\times g$ for 5 h at 4°C. Pellets were suspended in sample buffer, heated at 100°C for 5 min, and similarly stored frozen at –70°C prior to immunoblotting.

Purification of HDV particles. Plasma (4 ml) collected on week 7 postinoculation was clarified by centrifugation at 5,000 $\times g$ for 1 h at 4°C. The clarified plasma was layered on a 20% sucrose cushion in 150 mM NaCl–20 mM Tris hydrochloride (pH 7.4) (TNE) and sedimented at 38,000 rpm for 5 h at 4°C in an SW41 rotor. The pellet was resuspended in TNE buffer, and isopycnic centrifugation was performed in a 10 to 50% (wt/vol) CsCl step gradient in TNE buffer. The gradient was subjected to centrifugation at 38,000 rpm in an SW41 rotor for 18 h at 4°C. Fractions were collected from the bottom of the tube, and the density was determined by measurement of the refractive index. Samples of each fraction were used for DNA, RNA, and protein analysis. Prior to protein analysis, the samples were dialyzed against TNE buffer with a microconcentrator system (Centricon 30; Amicon Corp., Lexington, Mass.).

Detection of anti-HDAg antibodies. Antibodies to HDAg were detected by an enzyme-linked immunoassay (Anti-Delta EIA; Abbott Laboratories, North Chicago, Ill.).

RESULTS

To maximize the chance of successfully inducing an infection by transfection of chimpanzee liver with HDV cDNA, we chose a mixture of two recombinant HDV plasmids for the inoculum. The first, plasmid pSVL-D3, is capable of directing the transcription of a linear trimeric head-to-tail HDV RNA of genomic polarity. The second, plasmid pSVL-Ag, is capable of directing the synthesis of an antigenomic RNA spanning the entire HDAg open reading frame, thereby allowing the direct translation of HDAg. The rationale for this experimental approach was to provide the transfected cells from the outset with a means of HDAg production, should the efficient initiation of HDV RNA replication require this protein.

One week before inoculation and weekly thereafter, bleedings were performed for evaluation of HDV and HBV markers as well as serum aminotransferase levels. Between weeks 6 and 7 following inoculation, a large increase in

FIG. 1. Summary of serological markers in the inoculated chimpanzee X136. HDV RNA was detected by RNA blot hybridization, anti-HDAg antibodies were measured by an enzyme-linked immunoassay, and HDAg was detected by immunoblot. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels are expressed in international units per liter.

serum liver-specific enzymes occurred, indicative of severe liver damage (Fig. 1). Alanine aminotransferase and aspartate aminotransferase levels peaked on week 7 at 940 and 210 U, respectively, and returned to normal values (50 and 15 U, respectively) by week 12.

Total cellular RNA was extracted from liver biopsy specimens obtained on a weekly basis and analyzed for the presence of HDV-specific RNA by blot hybridization after gel electrophoresis and transfer to nitrocellulose. The first sign of infection occurred at week 5 by the appearance of viral RNA in the liver. HDV RNA was most abundant in the liver on weeks 6 and 7 and was no longer detectable on week 11. High levels of genomic HDV RNA were detected with an electrophoretic mobility corresponding to approximately 1.7 kb (Fig. 2A). In addition, we detected a low level of HDV RNA sequences of higher-molecular-weight, 3.5 kb in apparent length, which probably represent replicative dimeric forms of the genome. Several species of lower-molecular-weight RNA, 0.7 kb in length and smaller, may include candidates for the HDAg mRNA (9). Serum samples were examined for the presence of HDV RNA by the same procedure. The 1.7-kb form of the viral genome was detected in the serum at weeks 6 and 7, with the highest levels being present at week 7 (Fig. 2B).

An immunoblot analysis was made of the biopsy and serum samples. HDAg appeared simultaneously in both the liver and serum on weeks 6 and 7 (Fig. 3). Two distinct polypeptides with apparent molecular masses of 22 and 24 kilodaltons were observed in immunoblots, although only the smaller was predicted from the nucleotide sequence of the pSVL-D3 cDNA clone (18, 19).

To further characterize the HDV RNA and HDAg present in the serum, viral particles were sedimented from 4 ml of serum collected on week 7 after inoculation and were subjected to isopycnic centrifugation on CsCl. Each fraction was assayed for density and for the presence of HDAg, HDV RNA, and HBV DNA. Particles containing HDV RNA had a buoyant density of 1.27 g/cm³ (Fig. 4B), whereas particles containing HBV DNA displayed the characteristic density of

FIG. 2. RNA blot hybridization analysis of HDV RNA extracted from liver biopsy specimens and serum. Liver biopsy specimens (A) and serum samples (B) were taken 2, 3, 5, 6, 7, 9, 11, and 12 weeks after inoculation. (A) Total RNA (2 μ g) was isolated from each biopsy specimen, separated on a 1.5% agarose gel, and analyzed for the presence of HDV RNA after transfer to nitrocellulose and hybridization to an HDV-specific DNA probe. (B) RNA extracted from each serum sample (20 μ l) was analyzed by the same procedure. Radiolabeled marker RNAs (lane M) were generated with the radiolabeled RNA ladder system from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.).

mature HBV virions (Dane particles) of 1.28 g/cm³ (Fig. 4A). Fractions positive for HDV RNA were positive for HDAg also (Fig. 4C). These results indicate that infection resulted in synthesis and secretion of mature HDV particles.

The effect of HDV replication on the synthesis of HBV DNA replicative intermediates was examined (Fig. 5). Total cellular DNA was extracted from each biopsy sample and analyzed by the Southern blot hybridization technique. Transient suppression of HBV DNA replication was observed at the onset of clinical symptoms of HDV infection. Replicative forms of the HBV genome, relaxed circular, linear, and single stranded, were detected at much lower levels at weeks 6, 7, 9, and 11, corresponding to the maximum phase of HDV RNA replication (Fig. 5A). The level of HBV DNA from serum samples was analyzed by the same procedure, and a similar pattern of suppression of the level of virion-associated HBV DNA was observed during the peak of HDV infection (Fig. 5B). The level of HBsAg and HBV e antigen (HBeAg) present in the serum was examined by enzyme-linked immunosorbent assay with serial dilution of samples taken throughout the infection. No significant change in HBsAg titer was observed, but a small decrease (less than threefold) in HBeAg reactivity occurred at the time of maximum HDV replication (data not shown).

After resolution of the clinical symptoms of HDV infection, serum samples were analyzed for the presence of antibodies to HDAg by a commercial enzyme-linked immu-

FIG. 3. Immunoblot analysis of HDAg-related proteins extracted from liver biopsy specimens and serum. Samples were treated with 2% sodium dodecyl sulfate–2% β -mercaptoethanol and heated at 100°C. Immunoblots were analyzed by the method of Kuo et al. (19) with either liver biopsy (2 μ g protein) (A) or serum (1 μ l of serum) (B) taken 2, 3, 5, 6, 7, 9, 11, and 12 weeks after inoculation. The size markers, as indicated at the left side, were prestained proteins (Bethesda Research Laboratories). kDa, kilodaltons.

noassay. Seroconversion to anti-HDAg antibody positive was first detected 9 weeks after inoculation (Fig. 1).

DISCUSSION

We demonstrated that a cloned cDNA copy of HDV was infectious when injected directly into the liver of an HBV-infected chimpanzee. The resulting infection was characteristic of an experimental infection transmitted by parenteral inoculation of viral particles: the incubation period was similar to that of a virus-transmitted infection, and the appearance of high levels of HDV RNA and HDAg in the liver and serum followed kinetics typical of an acute infection (25, 29). The progeny virions had the characteristics of mature HDV particles including the proper density in CsCl, the presence of antigenic polypeptides with the molecular weight of HDAg, and HDV genomic RNA. Finally, we observed a marked, transient suppression of the synthesis of HBV DNA replicative intermediates during the active phase of HDV replication. This phenomenon has been described previously as a characteristic feature of HDV superinfection in both humans and chimpanzees (8, 13, 14, 16, 17, 29, 31).

Although this study clearly demonstrated that inoculation of cloned HDV cDNA directly into the liver was sufficient to initiate infection, we cannot make a conclusion about the requirement of a multimeric form of the HDV genome. In the viroid system, cloned DNA containing a head-to-tail multimeric copy of the genome is more efficient than a monomeric DNA genome for inducing replication in plants (10, 12, 41). It is believed that the DNA initially serves as a template for

FIG. 4. Analysis of serum-derived viral particles by CsCl gradient ultracentrifugation. Viral particles obtained from 4 ml of serum from week 7 postinoculation were subjected to centrifugation in a 10 to 50% (wt/vol) CsCl gradient for 18 h at 38,000 rpm in an SW41 rotor at 4°C. Fractions were collected from the bottom of the tube, and 1/10th of each fraction was used for DNA extraction, RNA extraction, and protein analysis, respectively. (A) DNA isolated from each fraction was separated on a 1.5% agarose gel and analyzed for the presence of HBV sequences after transfer to nitrocellulose and hybridization to an HBV sequence DNA probe. Radiolabeled fragments from *Hind*III-digested bacteriophage lambda DNA were used as markers. (B) RNA was isolated and separated on a 1.5% agarose–2.2 M formaldehyde gel and analyzed for the presence of HDV RNA after transfer to nitrocellulose and hybridization to an HDV-specific DNA probe. Radiolabeled marker RNAs were generated with the radiolabeled RNA ladder system from Bethesda Research Laboratories. (C) Proteins isolated from each fraction were analyzed by the immunoblot technique as described in the legend to Fig. 3. The numbering (2 to 15) of each fraction and its density are indicated in panel D. kDa, kilodaltons.

FIG. 5. DNA blot hybridization analysis of HBV DNA extracted from liver biopsy specimens and serum. Liver biopsy specimens (A) and serum samples (B) were taken 2, 3, 5, 6, 7, 9, 11, and 12 weeks after inoculation. (A) Total cellular DNA (2 µg) was isolated from each biopsy specimen, separated on a 1.5% agarose gel, and analyzed for the presence of HBV DNA after transfer to nitrocellulose and hybridization to an HBV-specific DNA probe. (B) DNA extracted from each serum sample (20 µl) was analyzed by the same procedure. Radiolabeled *Hind*III fragments of bacteriophage lambda DNA were used as markers (lane M). RC, Relaxed circular; L, linear; SS, single stranded.

DNA-directed RNA synthesis and that multimeric RNA molecules are formed and processed to circular forms of unit length which allow a subsequent RNA-directed pathway of replication to proceed (5, 6, 12). In contrast to HDV, viroid RNAs are strictly noncoding, and RNA-directed RNA synthesis is achieved either by an RNA-directed RNA polymerase that has been detected in plants (34) or by redirection of a host DNA-dependent RNA polymerase. The HDV genomic and antigenomic RNAs contain several open reading frames (21, 43, 44), but so far only one has been linked to an identified protein, HDAg. This protein is present with viral particles, and its properties include an RNA-binding activity that may be involved in the replication mechanism (7). The need for the coinfecting HDAg expression vector, pSVL-Ag, could not be addressed in this experiment. However, in a separate study, Kuo et al. (18) transfected cultured cells with the same recombinant clone, pSVL-D3, and obtained replication of the HDV genomic RNA. Those studies demonstrated that cotransfection with the HDAg clone, pSVL-Ag, was not necessary for genome replication and that genome replication was not dependent on the presence of HBV sequences. However, the present studies in an HBV-infected animal allow not only HDV genome replication but also the production of viral particles.

The ability of cloned viral DNA to initiate the replication of RNA viruses has been reported for different agents including bacteriophage Q β (42), poliovirus (26), and retroviruses (22), although in the latter case, a DNA copy of the

viral genome is involved in the natural cycle of replication. Several groups have also tested the infectivity of cloned DNA of different hepadnaviruses to determine the biological activity of recombinant DNA in this system (35, 39, 46). The direct introduction of DNA into the liver proved to be an effective route of transmission, but introduction of HBV DNA at sites other than intrahepatic was ineffective (45). On the basis of these results, we used the same approach for testing the infectivity of an HDV cDNA but did not test alternative routes of administration. Although unlikely in view of experiments conducted with HBV DNA, infection by intravenous inoculation should be considered a risk associated with manipulation of full-length HDV cDNA clones in laboratory work.

In a more detailed examination of the RNA in both the serum particles and the liver of our infected chimpanzee, we should be able to identify the occurrence of defective or variant genomes and study their structure and function. The high sequence variability observed among the different clones of HDV sequenced to date indicates that the viral genome can tolerate variability in its nucleotide sequence and perhaps in the size of the HDAg open reading frame (21, 44). Immunoblot assays have previously identified two distinct HDAg-specific polypeptides sometimes referred to as 24 and 27 kilodaltons in both liver and serum of HDV-infected patients (1, 2, 23, 33). Although a single 22-kilodalton HDAg polypeptide was predicted from the nucleotide sequence of pSVL-D3, two polypeptides (22 and 24 kilodaltons) bearing the delta antigen were identified by our immunoblot analysis. Several hypotheses can be proposed to explain this discrepancy. (i) A mutation in the HDAg-coding region may have introduced alternative translation initiation or termination sites; (ii) since HDAg can be phosphorylated (7), the difference in apparent molecular weight may represent different levels of phosphorylation; (iii) RNA processing may result in two distinct HDAg-specific mRNAs; or (iv) a type of posttranslational modification other than phosphorylation may account for the difference in molecular weight. A closer examination of the material derived from the infected chimpanzee may help clarify the relationship between the different electrophoretic forms and the predicted HDAg open reading frame.

This study demonstrates the relevance of using a cloned cDNA of HDV for functional analysis of the HDV genome. Recombinant plasmid pSVL-D3 contains a biologically competent clone that should be amenable to mutagenesis studies aimed at determining the role of specific regions of the HDV genome in the molecular biology and pathogenicity of this new viral agent.

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